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1) Crompton et al. Journal of General Virology. 1994; 75 (pt 1): 133-139.

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Targeted gene delivery by tropism-modified adenoviral vectors

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The utility of adenoviral vectors for gene therapy is currently limited due, in part, to the widespread distribution of the cellular receptor for the adenovirus fiber that precludes the targeting of specific cell types. In order to develop a targeted adenovirus, it is therefore necessary both to ablate endogenous viral tropism and to introduce novel tropism. We hypothesized that these two goals could be achieved by employing a neutralizing anti-fiber antibody, or antibody fragment, chemically conjugated to a cell-specific ligand. To test this concept, we chose to target the folate receptor, which is overexpressed on the surface of a variety of malignant cells. Therefore, we conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody. This Fab-folate conjugate was complexed with an adenoviral vector carrying the luciferase reporter gene and was shown to redirect adenoviral infection of target cells via the folate receptor at a high efficiency. Furthermore, when complexed with an adenoviral vector carrying the gene for herpes simplex virus thymidine kinase, the Fab-folate conjugate mediated the specific killing of cells that overexpress the folate receptor. This work thus represents the first demonstration of the retargeting of a recombinant adenoviral vector via a non-adenoviral cellular receptor.

Keywords: targeted adenovirus, gene therapy

Recombinant human adenoviral vectors of serotypes 2 (Ad2) and 5 (Ad5) have the ability to efficiently transfer genes to a range of cell types in vivo and have therefore been used in a number of gene therapy approaches[1,2]. However, the present generation of vectors suffers from the widespread distribution of the cellular receptor for the fiber of serotypes 2 and 5, which precludes the targeting of specific cell types[3,4]. Hence it is not currently possible to exploit the full potential of the adenovirus as a gene delivery vehicle exhibiting systemic stability following intravenous administration[3,5]. It is therefore desirable to alter the tropism of the adenoviral vector to permit gene delivery specifically to certain target cell types. This will require both the ablation of endogenous viral tropism and the introduction of novel tropism.

The initial high affinity binding of Ad2 and Ad5 to an as yet unidentified primary cellular receptor is mediated by the knob domain of the fiber capsid protein, as demonstrated by the ability of recombinant knob or anti-knob antibodies to inhibit adenoviral infection[6,7]. We hypothesized that it would be possible to ablate endogenous adenoviral tropism with a neutralizing anti-knob monoclonal antibody (Mab), and that novel tropism could be introduced by conjugating a cell-specific ligand to this Mab or an antibody fragment. To test this concept, we chose to target the high affinity folate receptor (K_d 10^{-9} M), which is overexpressed on the surface of several malignant cell lines, including ovarian, lung, and breast carcinomas and brain tumors[8-11]. Therefore, we conjugated folate to the Fab fragment of a neutralizing anti-knob Mab. We demonstrate that a complex between this Fab-folate conjugate and an adenoviral vector carrying the luciferase reporter gene redirects adenoviral infection of target cells via the folate receptor at a high efficiency. In addition, when complexed with an adenoviral vector carrying the gene for herpes simplex virus thymidine kinase, the Fab-folate conjugate mediated the specific killing of cells which overexpress the folate receptor. This work represents the first demonstration of the retargeting of a recombinant adenoviral vector via a non-adenoviral cellular receptor.

Results

Generation of neutralizing anti-knob monoclonal antibodies. In order to restrict gene delivery exclusively to the target cells, it is necessary to prevent the interaction between the knob domain of the adenovirus fiber and the cellular receptor that plays the major role in the determination of adenoviral tropism[6,7]. Since the specific amino acid residues in the knob which recognize the cell surface receptor have not been identified, it is not possible to ablate this binding site by using genetic techniques such as site-directed mutagenesis. However, a neutralizing anti-knob Mab should be capable of blocking the primary interaction between the adenovirus fiber and its cognate cellular receptor. We rationalized that if such an antibody were chemically conjugated to a ligand recognizing a specific cell surface receptor, it should be possible to target the adenoviral vector to this novel receptor (Fig. 1).

To develop a neutralizing anti-knob Mab, hybridomas were generated by standard techniques after immunization of mice with intact Ad5 followed by two rounds of immunization with purified recombinant Ad5 knob. On the basis of its high affinity binding to recombinant Ad5 knob and its ability to neutralize Ad5 infection of HeLa cells (data not shown), one clone, designated 1D6.14, was chosen for further study and the Mab was

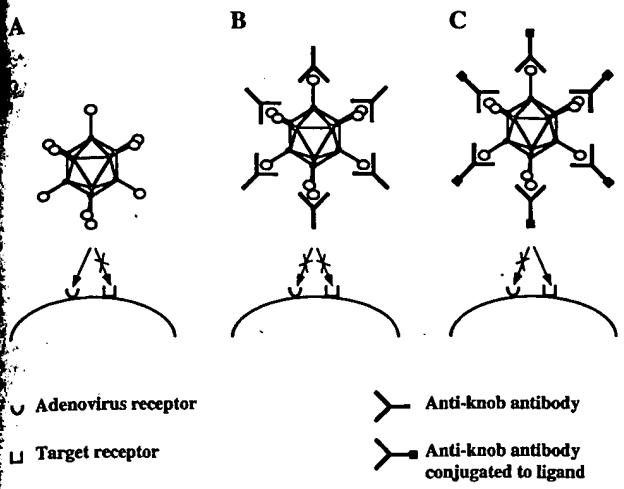

Figure 1. Strategy for retargeting an adenoviral vector. (A) Adenovirus binds to cells via the high affinity binding of the knob domain of the fiber to an as yet unidentified membrane surface receptor. (B) When complexed with a neutralizing antibody directed against the knob domain, the adenovirus is unable to bind to its cellular receptor. (C) Conjugation of a cell-specific ligand to the neutralizing antibody should permit binding to a novel target receptor on the cell surface.

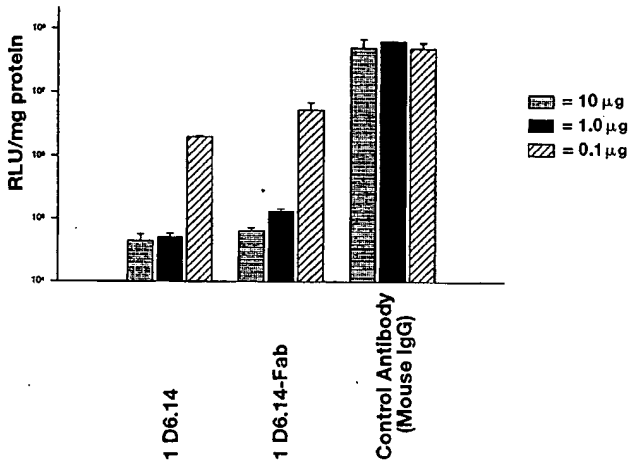

Figure 2. Anti-knob Mab 1D6.14 and its Fab fragment neutralize adenoviral infection. Varying dilutions of intact anti-knob Mab 1D6.14, the Fab fragment or control antibody (mouse IgG) were incubated at room temperature with 10^8 particles of AdCMVLuc[4], an adenoviral vector that expresses firefly luciferase from the CMV promoter. The expression of luciferase activity in cells infected with this vector is directly proportional to the number of infecting virus particles. The data are expressed as relative light units per mg of cellular protein. Results are the mean of triplicate experiments.

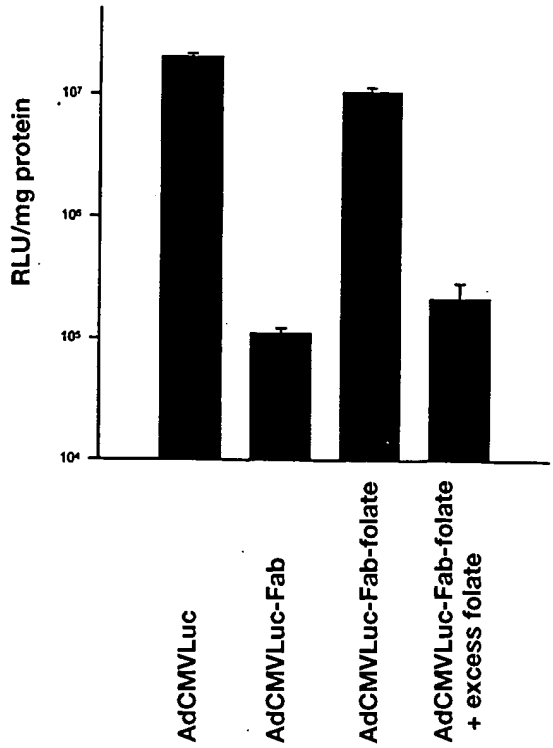

Figure 3. Retargeted adenoviral infection mediated by the Mab 1D6.14 Fab fragment-folate conjugate. The amount of 1D6.14 Fab or Fab-folate that gave maximum inhibition of infection by 10^8 particles of AdCMVLuc was determined. This optimal dose (0.5 μg) of Fab or Fab-folate was then incubated with 10^8 particles of AdCMVLuc. Results are the mean of triplicate experiments.

purified from ascites fluid by affinity chromatography using an immobilized protein A column.

Generation and analysis of Fab fragment of neutralizing anti-knob antibody. For the purposes of developing a targeted adenoviral vector by immunological methods, it would be preferable to use the Fab fragment of the antibody, rather than the intact immunoglobulin. By using the Fab fragment, we sought to prevent the two antigen-binding arms of the parent antibody from crosslinking different viruses to form large complexes that might prove refractory to cellular uptake. Intact 1D6.14 was digested with papain and the Fab fragments were purified. Both the parent antibody, 1D6.14, and the Fab fragment were capable of neutralizing adenovirus infection in a dose-dependent manner, whereas a control antibody failed to block infection (Fig. 2).

Construction and characterization of Fab-folate conjugate. The second requirement for a targeted adenoviral vector is the ability to recognize specific receptors expressed on the surface of the target cells. We conjugated the vitamin folate to the Fab fragment of the neutralizing anti-knob Mab, with the aim of targeting adenoviruses to the high-affinity folate receptor that is overexpressed on a number of malignant cell lines. Folate can be conjugated via its γ-carboxylate group to a variety of macromolecules, including antibodies, without losing affinity for its cellular receptor[12–15]. This has been exploited for the specific delivery of folate conjugates into folate receptor-positive cells[12–15]. Since folate and folate-macromolecule conjugates are internalized by the folate receptor by a mechanism termed potocytosis[16], which involves nonclathrin-coated caveolae with a diameter of 60 nm, an adenovirus (diameter 65–80 nm, excluding the fibers) would be too large to enter the cell by this pathway. After binding specifically to the cell surface folate receptors, the adenoviral vector should still be able to internalize via its native endocytotic pathway[17–19] mediated by the interaction of the penton base with secondary host cell receptors, av integrins[20,21].

Carboxyl groups of folate were coupled to amine groups of the Fab fragment of Mab 1D6.14 by a carbodiimide procedure[14]. The resulting conjugate, referred to as the Fab-folate conjugate, was characterized both structurally and functionally. The conjugation of folate to the antibody fragment was verified by SDS-PAGE under denaturing conditions followed by immunoblot analysis

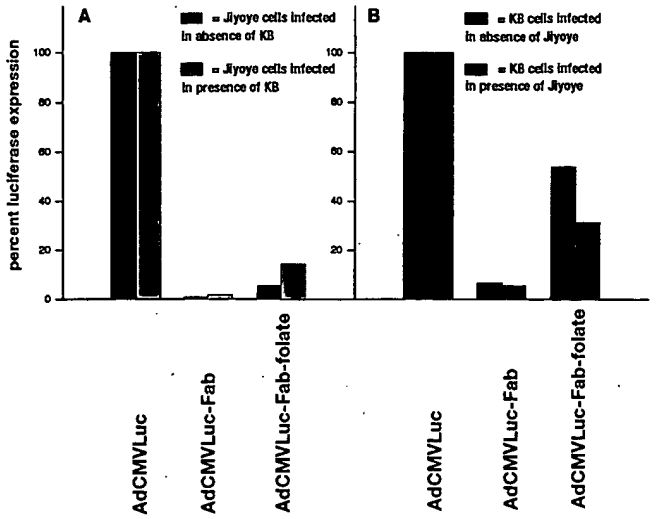

Figure 4. Targeted adenoviral infection of cells that overexpress the folate receptor. The optimal neutralizing amount (0.5 μg) of Fab or Fab-folate was incubated with 10^8 particles of AdCMVLuc at room temperature in a total volume of 20 μl HBS. (A) The infection of Jiyoye cells in the absence or presence of KB cells. (B) The infection of KB cells in the absence or presence of Jiyoye cells. Results are expressed as a percentage of the luciferase activity (relative light units per mg of cellular protein) achieved by infection of the target cells with the unmodified virus. Results are the mean of triplicate experiments.

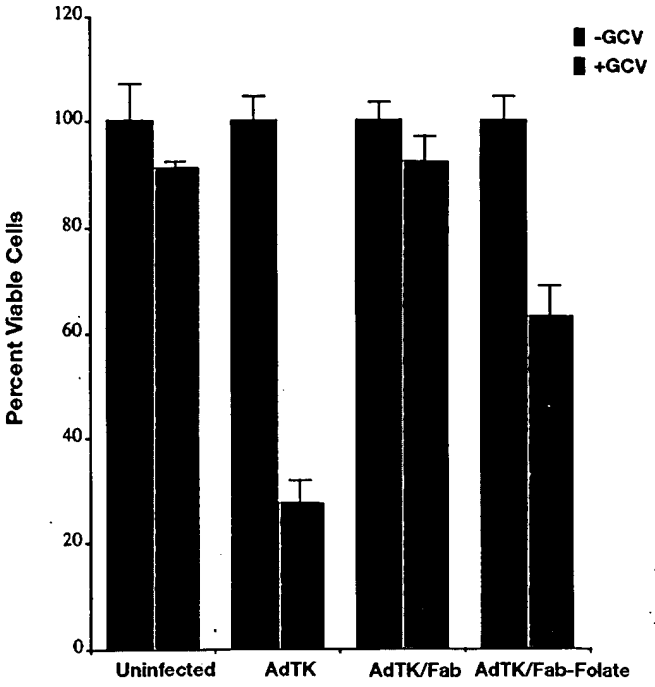

Figure 5. Targeted killing of cells by an adenoviral vector redirected via the folate receptor.

using an anti-folate Mab. An alkaline phosphatase-conjugated secondary antibody, specific for the Fc region of mouse IgG, was used to prevent cross-reaction with the Fab fragment of 1D6.14. The anti-folate antibody reacted specifically with the Fab-folate conjugate, while failing to recognize the unconjugated Fab fragment, thus confirming the conjugation (data not shown).

The ability of the Fab-folate conjugate to recognize the folate receptor was evaluated in a competition binding assay using ^{3}H-labeled folate and KB cells, a folate receptor-positive human nasopharyngeal carcinoma cell line[22]. Binding of the labeled folate to KB cells was inhibited by the Fab-folate conjugate and by a conjugate of folate with the intact 1D6.14 antibody, but not by the antibody alone (data not shown). Thus, the conjugation of folate to the Fab fragment of the neutralizing antibody had not destroyed the ability of folate to bind to its receptor.

We then determined whether the conjugation of folate to the neutralizing anti-knob Fab fragment had affected its ability to block adenovirus infection. AdCMVLuc[5], an E1-, E3-deleted Ad5 vector, which expresses firefly luciferase from the cytomegalovirus (CMV) promoter, was premixed with various concentrations of the Fab-folate conjugate prior to infection of HeLa cell monolayers. Expression of luciferase activity in infected cells was determined 24 hours postinfection, giving a value that is directly proportional to the number of infecting virus particles. The Fab-folate conjugate was capable of neutralizing adenoviral infection (data not shown). This neutralization was dose-dependent, with maximal inhibition of 10^8 viral particles occurring with 0.5 μg Fab-folate. This concentration was used in all subsequent studies.

Targeting of AdCMVLuc to folate receptor. To test the hypothesis that the Fab-folate conjugate could modify the tropism of an adenoviral vector, the vector AdCMVLuc was premixed with the optimal neutralizing concentrations of the unconjugated Fab, or the Fab-folate conjugate, prior to infection of KB cell monolayers maintained in folate-free medium. Luciferase activity was assayed 24 hours postinfection (Fig. 3). Greater than 10^7 relative light units were expressed per milligram of cellular protein upon AdCMVLuc infection. In contrast, the unconjugated Fab fragment blocked AdCMVLuc infection of KB cells by preventing the knob domain of the virus fiber from binding to its cellular receptor, resulting in a 99% inhibition of luciferase expression. A high level of luciferase activity was restored when AdCMVLuc was premixed with the Fab-folate conjugate, indicating that the retargeted virus was capable of efficient infection. When a competition experiment was performed in which the target cells were preincubated in folate-containing medium and the infection carried out in the presence of excess free folate, the Fab-folate conjugate failed to mediate infection of KB cells by AdCMVLuc. The free folate saturated the target receptor, preventing the binding of the viral complex. The Fab-folate conjugate was therefore capable of redirecting adenoviral infection of target cells specifically via the folate receptor.

To confirm that the Fab-folate conjugate could target adenoviral infection of KB cells in the presence of a second cell line that lacks, or expresses low levels of, the folate receptor, we used a suspension EBV-transformed B cell line, Jiyoye, that is transducible by recombinant adenoviral vectors[23]. Jiyoye cells failed to bind ^{3}H-labeled folate, indicating that the folate receptor was not expressed at a significant level (data not shown). The adenoviral vector AdCMVLuc was premixed with the optimal neutralizing concentrations of the unconjugated Fab fragment or the Fab-folate conjugate prior to infection of KB cell monolayers, suspension Jiyoye cells, or a 50:50 mixture of KB and Jiyoye cells maintained in folate-free medium. Since Jiyoye cells grow in suspension, while KB cells are adherent, the folate receptor-negative and receptor-positive cell types could be separated prior to the luciferase activity assay. Infection of Jiyoye cells by AdCMVLuc was blocked by the Fab fragment of the neutralizing Mab and was not restored when the virus was premixed with the Fab-folate conjugate (Fig. 4A).

In contrast, when maintained alone or in the presence of folate receptor-negative Jiyoye cells, KB cells could be efficiently infected by AdCMVLuc that was retargeted to the folate receptor by the Fab-folate conjugate (Fig. 4B). Therefore, the Fab-folate conjugate was capable of specifically mediating adenoviral infection of target cells that overexpress the folate receptor.

Targeted cell killing by tropism-modified adenoviral vector. In order to test the ability of the Fab-folate conjugate to redirect toxin-mediated cell killing, an E1-deleted Ad5 vector that expresses the prodrug-activating herpes simplex virus thymidine kinase (HSV-TK) gene[24] from the CMV promoter[25] was used for infection of KB cells (Fig. 5). Infection with AdCMVHSV-TK, and subsequent treatment with ganciclovir (GCV), resulted in 73% cell death. In contrast, when AdCMVHSV-TK was mixed with the neutralizing Fab only 8% of the KB cells were killed, indicating nearly 90% inhibition of TK/GCV mediated cell killing due to neutralization of adenoviral binding. Retargeting of the AdCMVHSV-TK vector with the Fab-folate conjugate restored TK/GCV mediated killing of almost 40% of the total cell population. This retargeting was specific for folate. An excess of folate, added to the AdCMVHSV-TK/Fab-folate infection media, resulted in an inhibition of cell death comparable to that seen with the Fab alone. Thus, not only could TK/GCV mediated cell killing be ablated with the neutralizing Fab, but retargeting of the virus via Fab-folate successfully overcame this inhibition and resulted in cell specific eradication.

Discussion

We have previously shown that a vector derived from Ad5, which possessed chimeric fibers composed of the tail and shaft domains of Ad5 and the knob domain of Ad3, specifically targeted the Ad3 cellular receptor[26]. This demonstrated that it is possible to alter Ad5 receptor recognition and provided support for the idea that it will be feasible to develop adenoviral vectors capable of targeted gene delivery to cells possessing specific surface receptor molecules. By complexing AdCMVLuc with Fab-folate, we have shown that viral infection of target cells is specifically redirected via the folate receptor, resulting in a level of gene transfer comparable to that achieved by native adenoviral infection. This is in marked contrast to the inefficient infection exhibited by retargeted retroviral vectors[27–30]. Infection of target cells via the folate receptor could be accomplished in the presence of folate receptor-negative cells. Furthermore, we have provided evidence of this targeting strategy for cancer gene therapy by demonstrating that the Fab-folate conjugate can modify the tropism of AdCMVHSV-TK to achieve the killing of tumor cells expressing the folate receptor.

Since the adenoviral particles targeted to the folate receptor in this study are too large to be accommodated within caveolae, the viruses could not have been internalized by potocytosis, the mechanism by which folate-macromolecule complexes enter cells[16]. Therefore, the demonstration that the Fab-folate conjugate was able to redirect adenoviral infection specifically via the folate receptor implies that modification of the first step of Ad infection, attachment of the knob domain of the fiber to primary cell surface receptors, does not affect the ability of the virus to accomplish the second step of infection, internalization. This finding supports our previous study showing that binding-incompetent adenovirus facilitates molecular conjugate-mediated gene transfer by the receptor-mediated endocytosis pathway[31]. In that work, the adenovirus was used to confer upon the molecular conjugate the ability to achieve endosomal lysis, thereby avoiding degradation within the lysosomes. However, the introduction of the adenovirus undermines the potential for cell-specific targeting via the ligand domain of the molecular conjugate vector. We overcame this problem by treating the adenovirus with a neutralizing antifiber antibody to prevent recognition of the adenovirus cellular receptor. Cell-specific delivery of the DNA component of the molecular conjugate was then achieved as a result of targeting by the ligand domain, with endosomal escape being effected by the adenovirus component. This demonstrated that binding of the adenovirus to its native receptor is not a prerequisite for adenoviral-mediated endosome disruption[31]. The processes of adenoviral binding and subsequent entry steps are not functionally linked. This result suggests that the range of cell-targeting ligands which can be employed in the construction of tropism-modified Ad vectors need not be restricted by the native internalization pathway of the ligand.

It is possible to generate tropism-modified adenoviral vectors capable of targeted cell-specific gene delivery via a non-adenoviral receptor. This is a step towards our goal of developing a targeted adenoviral vector capable of the high efficiency infection of specific cell types after intravenous administration. Other types of vector, such as retroviral vectors and molecular conjugates, are less suitable for in vivo gene delivery after systemic administration since they are highly labile in the presence of serum[32,33]. Therefore, a targeted adenoviral vector will enormously expand the potential therapeutic approaches that may be attempted employing gene therapy strategies.

Experimental protocol

Cells and viruses. HeLa, 293[34], KB, and Jiyoye cells were obtained from the American Type Culture Collection (Rockville, MD). HeLa, 293, and KB cells were maintained as adherent populations in DME/Ham's F-12 medium and Jiyoye cells were maintained in suspension in RPMI 1640 medium. The media were supplemented with 10% FCS, glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 mg/ml) and the cells were propagated at 37°C in a 5% CO_2 atmosphere. FCS was purchased from HyClone Laboratories (Logan, UT) and media and supplements were from Mediatech (Herndon, VA). Prior to each experiment to target the folate receptor, KB and Jiyoye cells were passaged twice in folate-free RPMI 1640 medium (ffRPMI; Gibco-BRL, Grand Island, NY) containing the above-mentioned supplements. AdCMVLuc[5], an E1-, E3-deleted Ad5 vector which expresses firefly luciferase from the CMV promoter, was generously provided by R.D. Gerard, University of Texas Southwestern Medical Center (Dallas, TX). AdCMVHSV-TK, an E1-deleted Ad5 vector, which expresses herpes simplex virus thymidine kinase from the CMV promoter, was constructed using homologous recombination techniques[25]. The recombinant adenoviruses were propagated on the permissive 293 cell line and purified[35].

Generation and characterization of neutralizing anti-knob Mab. Anti-knob Mabs were generated by established methods[36] after immunization of BALB/c mice with Ad5, followed by two rounds of immunization with purified recombinant Ad5 knob[6], a gift from R.D. Gerard. Sensitized lymphocytes were fused with P3-X63-Ag8.653 cells. The reactivity of the hybridoma supernatants with trimeric Ad5 knob was determined in an ELISA. The ability of the hybridoma supernatants to neutralize Ad5 infection was assayed by endpoint CPE.

Purification of anti-knob Mab and Fab fragment. The 1D6.14 hybridoma cells were injected into BALB/c mice and ascites fluid collected[36]. Purification of the Mab was performed by affinity chromatography on immobilized protein A using an ImmunoPure IgG purification kit (Pierce, Rockford, IL). Fab fragments were prepared and purified by digestion of 1D6.14 on immobilized papain followed by affinity chromatography on immobilized protein A, using an ImmunoPure Fab purification kit (Pierce). After extensive dialysis against phosphate-buffered saline (PBS) the concentrations of the purified Mab and Fab fragment were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Conjugation of folate to 1D6.14-Fab. Carboxyl groups of folate were coupled to amine groups of intact Mab 1D6.14, or the Fab fragment, by a carbodiimide procedure, as described[14]. Folate (5.0 mg, 11.3 μmol; Sigma, St Louis, MO) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride, EDC, (10.8 mg, 56.5 μmol; Pierce) were dissolved in dimethyl sulfoxide (1.0 ml) and incubated at room temperature in the dark for 30 min. A 100-fold molar excess of this activated folate was added to the intact IgG or Fab fragment in PBS and incubated at room temperature for 1 h. Excess folate was removed by ultrafiltration using a Centricon membrane with a 30 kD molecular weight cut off (Amicon, Beverly, MA). After

four washes with PBS, conjugates were collected from the membranes and stored at 4°C.

[^{3}H]-folate-binding assay. Competition binding assays were conducted in triplicate using [^{3}H]-folate (Amersham, Arlington Heights, IL; specific activity = 47 Ci/mmol) and KB cells rinsed well with PBS to remove excess folate present in the culture medium. The competitors (50 μg of 1D6.14, 1D6.14-folate, 1D6.14-Fab-folate or folate in 100 μl PBS) were added to 1×10^6 KB cells in 100 μl PBS, followed immediately by the addition of [^{3}H]-folate (100 μl , 2×10^5 cpm). After incubation at room temperature for 1 h, the cells were washed with 4 ml of PBE (1% BSA, 0.2 M EDTA in PBS) and centrifuged at 1720 g for 10 min. The wash was then aspirated and cell-associated radioactivity determined in a scintillation counter.

Antibody neutralization of adenovirus infection. Varying dilutions of intact anti-knob Mab 1D6.14, the Fab fragment or the Fab-folate conjugate were incubated with 10^8 particles of AdCMVLuc at room temperature in a total volume of 20 μl HBS. After 30 min, the volume was increased to 1 ml with DMEM/F-12 + 2% FCS and the complexes were added to 6-well plates containing 1×10^6 HeLa cells previously rinsed with PBS. After incubation for 24 h at 37°C, the cells were lysed and extracts were assayed for luciferase activity using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol.

Targeting of AdCMVLuc to the folate receptor via 1D6.14 Fab-folate. 1D6.14 Fab or Fab-folate (0.5 μg) was incubated with 10^8 particles of AdCMVLuc at room temperature in a total volume of 20 μl HBS. After 30 min, the complexes were diluted to 1 ml with ffRPMI + 2% FCS and added in triplicate to 6-well plates containing 1×10^6 KB or Jiyoye cells which had been washed with PBS. After incubation for 24 h at 37°C, the cells were lysed and extracts assayed for luciferase activity as described above. In experiments involving a mixed populations, 5×10^5 KB and Jiyoye cells were used. The suspension Jiyoye cells were separated from the adherent KB cells prior to the luciferase assays. For folate inhibition studies, KB cells were preincubated for 30 min at room temperature in 3 ml ffRPMI + 10% FCS containing 50 μg folate, and the AdCMVLuc-Fab-folate complex was added to the cells in 1 ml ffRPMI + 2% FCS containing 50 μg folate.

Cell killing. KB cells were plated in 96-well plates at 10,000 cells per well. The following day, the cells were either left uninfected or treated at an moi of 10 with 2×10^6 pfu AdCMVHSV-TK alone or complexed with 1D6.14 Fab, Fab or Fab-folate as described above (n=8). Half of the samples were treated 24 h postinfection with ffRPMI + 10% FCS containing the prodrug GCV at a concentration of 20 μM; the remaining cells were given only ffRPMI + 10% FCS. Cell viability was determined 6 days later using a colorimetric cell proliferation assay (Cell Titer 96 Aqueous Non-radioactive MTS Cell Proliferation Assay; Promega). Briefly, 20 μl of assay mixture were added to each well of cells and the plates incubated for 1–4 h at 37°C before absorbance was measured at 490 nm in a 96-well plate reader (Molecular Devices, Menlo Park, CA). In these experiments KB cells were plated on the day of the assay in ffRPMI to generate a standard curve. Cells were removed by trypsinization and plated in triplicate wells at the following densities: 50,000; 20,000; 10,000; 5,000; 2,000; and 0 cells per well. From the standard curve, viable cell numbers could be calculated for experimental groups using the SOFTmax computer software (Molecular Devices).

Acknowledgments

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